Lasers for Molecular Fluorescence

Advantages in time resolution, sensitivity and speed lead to an ultimate hope: watching cells in living color

BY DIETRICK E. THOMSEN

Things light up better with lasers—a lot of things. One of them, it seems, is molecular fluorescence. As pointed out last week, fluorescence has been used for a long time to tell scientists where things are. Physicists and chemists have used it to learn about the structure of atoms and molecules and the dynamics of atomic and molecular processes. Biologists have used it as a tracer.

All this comes about because particular molecules (and atoms, too) will absorb light of a specific color and almost immediately emit light of a different specific color. You can thus tell what it is and where it is. To a physicist or chemist there is much more. The energy absorption produces an energetically excited state, and since physical systems tend to return to the lowest possible energy state as soon as they can, the emission removes the excited state. The colors involved in fluorescence and the time it takes are determined by the structure of energy states in a given molecule and by its environment, so a lot can be learned if the required data can be gotten out.

Lasers as fluorescence exciters can provide more opportunity than can the traditional flashlamps. They offer advantages in sensitivity, selectivity and time resolution. As an example of sensitivity improvement, a report by Jeffrey H. Richardson, S. M. George and M. E. Ando of the Lawrence Livermore Laboratory mentions a one-to-four-orders-of-magnitude increase in detection of organic molecules in aqueous solution. As an example of measurements not possible before, Gilbert R. Haugen of Livermore is engaged in an attempt to measure fluorescence lifetimes on the subnanosecond (less than a billionth of a second) level, and possibly as fast as they occur.

To reach capabilities of this kind, 10,000-fold increases in certain sensitivities and time resolutions in the nanoseconds, the LLL group, led by Richardson, has been developing pumped dye lasers for inducing fluorescence. The latest and, they think, best and most versatile system, uses a krypton ion laser as a pump. In the original version violet emission from the krypton excites a coumarin dye laser that is tunable over the blue-green range of the spectrum. The system is mode locked; that is, the vibrations of the two lasers are made to work together so that the pulses that come out of the dye laser are much shorter than those that come from the krypton laser. Fifty-picosecond pulses from the krypton become 1- to 20-picosecond pulses from the coumarin.

The pulses have to be short to avoid overlapping the fluorescent emissions and washing them out of the detectors, they have to come at a fast repetition rate (88 million pulses per second is the maximum here) to build up a detectable fluorescence signal in a short time, and they have to be gentle so as not to alter the chemistry they are probing—"fractions of a nanojoule per pulse," says Richardson. The tunability is especially necessary for making laser systems practical for a wide range of work in molecular fluorescence. The advantage of krypton, says Richardson, is that it has so many spectral lines that they can be used one by one to excite different dyes with different ranges of tunability. The total system will be tunable from 3,800 to 9,000 angstroms, which is all the way across the visible spectrum and into the infrared.

Previous laser systems for molecular fluorescence had worked only in red light.

Photos: John H. Douglas

Michael A. Rivelli

So here the first interest was in producing a tunable blue-green system for use on compounds that absorb blue. An example is benzo(a)pyrene (BaP), a chemical found in smoke. When BaP enters the body, an enzyme called aryl hydrocarbon hydroxylase turns it into a potent carcinogen, a diol-epoxide of BaP. The latter molecule binds to DNA and can sometimes cleave it, an effect that alters the genetics of the cell. "One of our goals is to use fluorescence to monitor the binding of diol-epoxide BaP to DNA *in vivo*," Richardson writes.

Another subject of study is the common biological dye rhodamine 6G. The idea is to be able to use the rhodamine 6G to label large molecules in biology and study immunochemistry and genetics. DNA is one example. The intention is to study the kinetics and binding of these molecules and the effects of the local environment, especially of the fluid, in which they are immersed.

Richardson and Sam Brown of Purdue have a photoelectrochemistry project in which they wish to study the transient effects that follow irradiation of electrodes in solution. Electrodes are substances that give off electrons when they are irradiated. The experimenters are looking at the scavenging of the electrons by organic molecules and they hope to be able to learn basic phenomena behind the effect and perhaps how to enhance it for practical use (in solar cells, for instance). "Chlorophyll and benzoquinone have been suggested as a photogalvanic cell," Richardson says, "and we're looking now at the dynamics involved on a very short timescale. We're starting off now at nanoseconds."

Having demonstrated that lasers increase the sensitivity of fluorescence measurements, he now wants his group to concentrate on improving the selectivity of laser methods, their ability to tell one

molecule from another. The major problem is spelled out by Lawrence W. Hrubesh: "Even in the gas phase there is quite a broad band of absorption and emission [from a given molecule]." That is, the characteristic wavelengths are not single and sharp, but occupy a short range or band. The bands of different substances may overlap to some extent and create confusion.

Within the broad band is a fine structure, a series of details relating to such things as vibrational motion of the molecule. These details differ from molecule to molecule. Hrubesh thinks it might be possible to excite these vibrational details with a longer wavelength than Richardson and others have been using, light from a carbon dioxide laser, which is in the infrared at about 10 microns. By a kind of double resonance both the fluorescence and the vibrations could be excited, thus enabling the investigators to tell one molecule from another with much greater accuracy.

Hrubesh has already used infrared lasers in molecular identifications of high sensitivity and great specificity. In this case the laser excites not fluorescence but rotational motions of gas molecules, and Hrubesh studies the radiation given off as these rotations give up their energy. The result is a method for determining the presence of extremely minute amounts of trace compounds in a gas sample. It can be used to analyze air pollutants, for example, and some day Hrubesh envisions that it may be used to monitor metabolites exhaled by a person standing in a special cubicle.

Fluorescence has long been of interest to chemists. It can enable them to study chemical reactions on the fly. One of the experimental programs in Richardson's group involves a combination of laser-induced fluorescence and chemiluminescence to study the reactions of alkali earth metal compounds with oxidants. One reason for studying these particular compounds, says Michael A. Rivelli, is that many of them are considered candidates for chemical lasers.

One of the features of this series of experiments is that the compounds start out in an energetically excited state and not in the ground state, which is the usual way chemical reactions are started. The metal compound is boiled and vaporized in a crucible at the bottom of a special cell. As the vapor rises through an inert carrier gas, the excited state is induced by the application of an electric field or laser light. Then a ring at the top of the cell introduces the oxidant, and the reaction goes on. A colorful chemiluminescent flame appears. This is a cold flame. The energy changes that produce the light are not connected to the sort of vibrations measurable as heat.

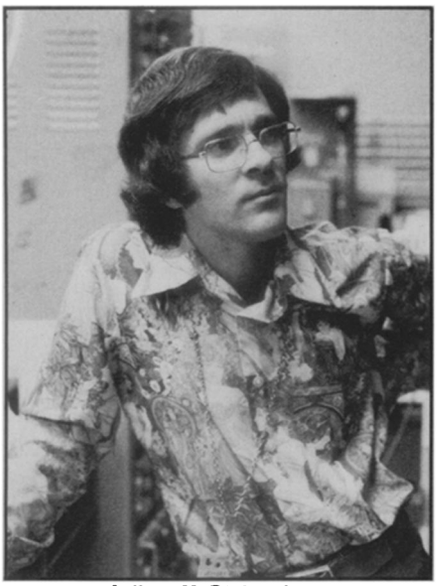
The chemiluminescence is analyzed to learn about the reaction products, but meanwhile laser induced fluorescence is used to study the intermediate states of the reactants. Thus the group can learn about the reaction step by step. And what they learn about atomic spectroscopy as they pick off intermediate states is applicable elsewhere. Richardson and Rivelli give the example of the products of radioactive decay. The work that led to the new radioactive decay method for determining the age of the universe that was made public about two years ago (SN: 7/10/76, p. 19) depended in some aspects on this kind of experiment. "Lasers are neat because they provide sensitivity and selectivity unequaled by conventional techniques," says Rivelli.

Lasers also improve the taking of data. With a laser, according to Haugen, more energy comes out per pulse and the energy comes out faster. This facilitates the time-correlated counting of single photons that he and his collaborators are engaged in. The data build up a signal-to-noise ratio from which good information can be extracted much more rapidly. One of the things Haugen and co-workers are trying to do is measure the lifetime of the fluorescent excited state in subnanoseconds. With lasers producing picosecond pulses they may be able to do it. The lifetime is the datum particularly affected by changes in the environment of fluorescent molecules, and if it takes an hour of counting photons to get a signal-to-noise ratio that's any good, a biological system, for example, can change a lot.

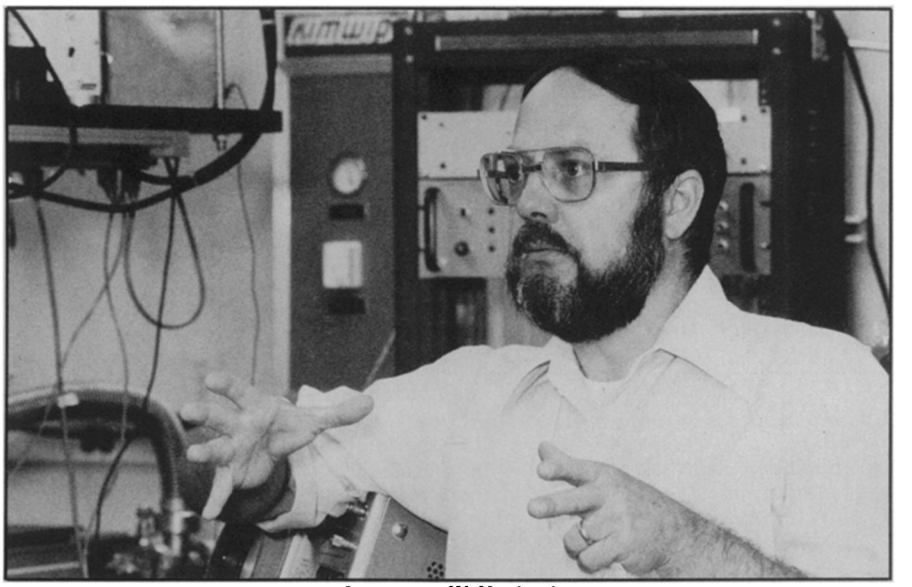
Space is another problem in molecular fluorescence. Specimens are so small and the equipment is so big. But laser light is spatially coherent. "We can examine small regions of space—which you can't do with a flashlamp," says Haugen. "Eventually we'd like to look at a single cell and do a lifetime measurement inside the cell."

The ultimate project here is an attempt to measure fluorescence lifetimes in real time. The experiments employ an interference technique. The laser has 35 modes of vibration that are allowed to beat against each other. This produces a spectrum of beat frequencies. Although the laser frequencies are optical, the sum frequencies that result from adding the modes together are in the radio range. Irradiating the fluorescent material with these radio frequencies serves as a kind of filter. It modulates the fluorescence so that the data that come out are the inverse, more or less, of the fluorescence lifetime, and so the lifetime can be easily found.

If the method works, it will permit such neat tricks as measuring the fluorescence lifetime of a molecule dissolved in a falling water drop — while the drop falls — or a fluorescent equivalent of flash photolysis in which chemical changes induced by light are followed in real time, or the following of chemical processes as they might occur in flowing liquids or inside cells. □



Jeffrey H. Richardson



Lawrence W. Hrubesh